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1    **Development of a parallel reaction monitoring-MS method to quantify**  
2    **IGF proteins in dogs: and a case of non-islet cell tumor hypoglycemia**

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13

14    **Abstract**

15    Non-islet-cell tumor hypoglycemia (NICTH) is a rare paraneoplastic phenomenon well described in dogs  
16    and humans. Tumors associated with NICTH secrete incompletely processed forms of insulin-like growth  
17    factor-II (IGF-II), commonly named big IGF-II. These forms have increased bioavailability and interact with  
18    the insulin and IGF-I receptor causing hypoglycemia and growth-promoting effects. Immunoassays  
19    designed for human samples have been used to measure canine IGF-I and -II, but they possess some  
20    limitations. In addition, there are no validated methods for measurement of big IGF-II in dogs. In the  
21    present study, a targeted PRM MS-based method previously developed for cats has been optimized and  
22    applied to simultaneously quantify the serum levels of IGF-I, IGF-II, and IGFBP-3, and for the first time,  
23    the levels of big IGF-II in dogs. This method allows the absolute quantification of IGF proteins using a  
24    mixture of QPrEST™ proteins previously designed for humans. The method possesses good linearity and  
25    repeatability, and has been used to evaluate the IGF-system in a dog with NICTH syndrome. In this dog,  
26    the levels of big IGF-II decreased by 80% and the levels of IGF-I and IGFBP-3 increased approximately 20  
27    and 4 times, respectively, after removal of the tumor.

28

29

30    **Keywords**

31    Big IGF-II, dog NICTH, IGF system, PRM, targeted proteomics.

## 32 INTRODUCTION

33 Non-islet-cell tumor hypoglycemia (NICTH) is a rare paraneoplastic phenomenon that is well described in  
34 both dogs and humans. NICTH denotes the syndrome of hypoglycemia produced by or associated with  
35 any neoplasm other than an insulinoma.<sup>1</sup> In most of the human cases, this syndrome is derived from an  
36 overproduction of insulin-like growth factor-II (IGF-II) by the tumor, including mature IGF-II or  
37 incompletely processed forms of IGF-II, that are referred to as big IGF-II.<sup>2-4</sup> In humans and animals, IGF-I  
38 and -II are both important regulators of growth and metabolism, and circulate bound to one of a family  
39 of six insulin-like growth factor-binding proteins (IGFBP 1-6), that modulate IGF-bioavailability.<sup>5</sup> Of the  
40 IGFBPs, IGFBP-3 is the most abundant in the circulation and transports more than 75% of IGF-I or -II in a  
41 ternary complex with an acid-labile subunit (ALS).<sup>6</sup> The large size of this complex (~ 150 kDa) limits the  
42 passage of IGFs across the capillaries,<sup>7</sup> thus prolonging their half-life from a few minutes, to hours or  
43 days, and determining their circulating concentrations.<sup>8</sup> IGFs and IGFBPs also form binary complexes (40-  
44 50 kDa) that are sufficiently small to cross the capillary membrane and gain access to most tissues.<sup>9</sup> In  
45 NICTH cases, big IGF-II can contribute up to 60% of the total circulating IGF-II concentration. It has been  
46 proposed that impaired proteolytic processing is due to the absence of glycosylation in the big  
47 isoforms,<sup>10</sup> as well as the exceeded proteolytic capacity of the tumor cells.<sup>11</sup> Moreover, although big IGF-  
48 II isoforms bind to IGFBPs with the same affinity as mature IGF-II, ternary complexes are formed less  
49 readily, and the formation of binary complexes is promoted.<sup>12,13</sup> Finally, some big IGF-II isoforms have  
50 reduced affinity for the IGF-II receptor, which may decrease its degradation and thereby increase  
51 bioavailability.<sup>14</sup> All of these effects increase the amount of bioavailable IGF-II and big IGF-II, that can  
52 interact with the insulin and IGF-I receptor causing hypoglycemia and growth-promoting effects.<sup>1,15</sup>

53 In normal circumstances, growth hormone (GH) induces synthesis of IGF-I and ALS by the liver. In  
54 addition, serum IGFBP-3 increases as a result of increased availability of ternary complex formation.<sup>16</sup> In

55 vitro studies demonstrate that IGF-I and IGF-II have inhibitory effects on pituitary GH.<sup>17</sup> Consistent with  
56 a negative feedback by big IGF-II forms in NICTH, patients have suppressed IGF-I, ALS and IGFBP-3 which  
57 increase in response to growth hormone treatment.<sup>18,19</sup> Total IGF-II may be either within the reference  
58 interval, decreased or increased in patients with NICTH.<sup>1</sup> Currently, reduced circulating IGF-I  
59 concentrations are routinely used in the investigation of suspected NICTH.<sup>1,20</sup> However, since IGF-I may  
60 be suppressed in severe catabolic illness,<sup>21</sup> measurement of big IGF-II is more specific for the disorder.  
61 Simultaneous measurements of IGF-I, IGF-II, big IGF-II and IGFBP-3 could aid in diagnosing NICTH as well  
62 as improving understanding of the GH-IGF-system in health and disease. To the best of our knowledge,  
63 the serum concentrations of big IGF-II protein in dogs have never been measured before.

64 Canine IGF-I, IGF-II and IGFBP-3 concentrations have been measured using a variety of methods.  
65 Radioimmunoassays (RIA) have been used to study the levels of IGF-I and -II,<sup>22-25</sup> enzyme-linked  
66 immunosorbent assays (ELISA) to measure the levels of IGF-I,<sup>26</sup> and Western Ligand Blotting (WLB) to  
67 evaluate the levels of IGFBP-3.<sup>22-23</sup> However, all these methods have limitations. The use of human  
68 immunoassays for analysis of samples from other species can result in weak reactivity or unwanted  
69 cross-reactivity. Moreover, IGFBPs can interfere with IGFs measurements, causing both false high or low  
70 values depending on the assay.<sup>27</sup> In addition to this problem, data available from the College of American  
71 Pathologists proficiency testing program for IGF-I demonstrated that interlaboratory variability of human  
72 IGF-I immunoassays has an RSD up to 33.5%,<sup>28</sup> and there is no available reference standard for  
73 quantification of canine IGF-II.

74 To solve these problems, and due to the good capabilities offered by liquid chromatography (LC) coupled  
75 to mass spectrometry (MS) in other fields,<sup>29</sup> MS-methods have been proposed to measure IGFs  
76 proteins.<sup>30</sup> In comparison to RIA, ELISA and WLB, LC-MS-methods require smaller volumes of samples  
77 and allow the unambiguous identification and quantification of multiple proteins in a single experiment.

78 Published studies have applied this methodology to quantify IGF-I,<sup>28,31-33</sup> or IGF-I, IGF-2, IGFBP-2 and  
79 IGFBP-3 simultaneously in humans,<sup>34</sup> as well as in other species.<sup>35,36</sup> In most of these experiments, and  
80 due to the wide dynamic range of protein concentrations, a tedious and/or time-consuming step of  
81 protein depletion is necessary to remove the most abundant proteins and to avoid the suppression of  
82 the signal of the least abundant. To achieve this, different protocols have been developed using  
83 acetonitrile,<sup>28</sup> SPE columns,<sup>32</sup> or specific antibodies for protein purification.<sup>31</sup> After sample preparation,  
84 the analysis of the proteins is often performed using a triple quadrupole MS spectrometer, where a  
85 peptide (precursor) is targeted for its fragmentation, and the quantification is performed at the MS/MS  
86 level using 3 to 6 transitions previously selected (commonly known as selected/multiple reaction  
87 monitoring (SRM/MRM)).<sup>37</sup> However, the development of more advanced mass spectrometers has  
88 enabled the use of other methods for quantification, such as the parallel reaction monitoring (PRM).<sup>38,39</sup>  
89 This method is mainly performed using quadrupole-Orbitrap mass spectrometers, where a precursor is  
90 selected, all transitions are measured, and the selection of fragments for quantification is done post-  
91 acquisition. Since full MS/MS spectra of the targeted peptides are acquired with high resolution and high  
92 mass accuracy, a PRM-based targeted method of protein quantification is highly selective and  
93 specific,<sup>40,41</sup> making it a very good method for targeted proteomics in complex matrices such as serum or  
94 plasma.<sup>42</sup> In addition, the great capabilities of this instrumentation have recently allowed the analysis of  
95 different IGFs in feline serum without extensive sample preparation.<sup>43</sup> In targeted MS-methods, heavy  
96 isotope-labelled peptides or full-length proteins are used for quantification of the endogenous proteins,  
97 which allow the comparison of their signals with the internal standards that are spiked into the samples  
98 during processing. The most commonly used peptides contain arginine or lysine with <sup>13</sup>C and <sup>15</sup>N isotope  
99 at the C-terminal end, and the most used synthetic proteins are QconCAT and PSAQ.<sup>44,45</sup> Other synthetic  
100 proteins are available, such as the QPreEST™ proteins.<sup>46</sup> QPreEST™ proteins are 50-150 amino acid-long  
101 segments of human proteins with heavy isotope-labelled (<sup>15</sup>N, <sup>13</sup>C) lysine and arginine, covering more

102 than 80% of the human protein-coding genes.<sup>47</sup> However, these proteins are rarely used to quantify  
103 human and non-human proteins.

104 To the best of our knowledge, no MS-based method has been published that quantifies canine IGF-I, IGF-  
105 II, big IGF-II and IGFBP-3 simultaneously. Therefore, the aim of this study is to optimize and use a  
106 previously targeted PRM MS-based method developed for cats, to absolutely quantify the levels of these  
107 IGF proteins and, for the first time, the levels of big IGF-II in dogs. The method was applied to diagnose  
108 and monitor a recent case of canine NICTH that was successfully managed surgically.

## 109 **MATERIALS AND METHODS**

### 110 **Chemicals and reagents**

111 Acetonitrile (ACN), formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Merck  
112 (Darmstadt, Germany). Ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>), urea, dithiothreitol (DTT) and iodoacetamide  
113 (IAA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). For the tryptic digestion, trypsin  
114 (sequencing grade modified, Promega, Madison, WI, USA) was used. Ultrapure water was prepared by  
115 the Milli-Q water purification system (Millipore, Bedford, MA, USA).

### 116 **Canine serum samples**

117 Healthy dogs (n=7) were sampled at the University Animal Hospital, Swedish University of Agricultural  
118 Sciences, Uppsala (Sweden), as well as in a smaller private clinic. All dogs were considered healthy by  
119 their owners and after a clinical examination by a veterinarian. Since weight is strongly related to IGF-I  
120 concentrations,<sup>48-49</sup> dogs were selected to represent a wide range of size. The breeds included Labrador  
121 Retriever, Australian Shepard, Russkiy Toy, German Shepard, Cane corso, Border Collie and Mixed breed.  
122 Age ranged from 1-9 years with median (IQR) of 4 (2-7) and weight between 2.4-46 kg with median (IQR)  
123 of 24 (14-36). There were 1 spayed female, 3 intact females and 3 intact males. The study was approved



124 by the Swedish Animal Ethics Committee (no. C193/14) and all owners provided written informed  
125 consent. Blood was drawn into plain tubes and centrifuged within 30-60 minutes. Serum was either  
126 frozen in -20°C or sent to the laboratory by post at ambient temperatures. All blood samples arrived  
127 within 24 hours after sampling. Blood samples from a dog with suspected NICTH syndrome, before and  
128 after tumor removal, were also used for validation.

## 129 **Protein standards**

130 To be able to match tryptic peptides in QPrEST™ proteins with dog sequences, Clustal Omega (1.2.4)<sup>50</sup>  
131 was used for sequence alignment of *Homo sapiens* and *Canis familiaris* amino acid (aa) sequences  
132 (**Figure 1**). QPrEST™-containing peptides matching with *Canis familiaris* IGF-II (Cat. Number 22489) and  
133 IGFBP-3 (Cat. Number 23429), with ≥99% isotopic purity and ≥80% peptide purity, were provided by  
134 Atlas Antibodies (Stockholm, Sweden). For IGF-I, the peptide GPETLCGAELVDALQFVCGDR synthesized  
135 with heavy-labelled (<sup>15</sup>N, <sup>13</sup>C) lysine and arginine (≥99% isotopic purity and ≥95% peptide purity) was  
136 purchased from New England Peptides (Gardner, MA, USA). The uniqueness of the sequences to the  
137 targeted proteins was checked using the “peptide search” tool of the Uniprot database against Canine  
138 proteins, and using Skyline.

## 139 **In-solution tryptic digestion**

140 The protein content in dog sera was measured using the Bradford protein assay (Bio-Rad Laboratories,  
141 Hercules, CA, USA). For normalization, a volume of sera that contained 30 µg of total protein was taken  
142 from each sample, and submitted to in-solution digestion. Firstly, the volume was adjusted with 0.4 M  
143 NH<sub>4</sub>HCO<sub>3</sub>, 1 M Urea, pH 8 to a total volume of 200 µL, and then the QPrEST™- containing peptides were  
144 spiked into the samples at different concentrations depending on the study (see below). The samples  
145 were sonicated for 3 minutes and after that, 10 µL of 45 mM DTT was added and the samples were kept  
146 at 50°C for 15 minutes to reduce the proteins. To irreversibly carbamidomethylate the cysteines, 10 µL of

147 100 mM IAA was added, followed by 15 minutes incubation at room temperature in darkness. For the  
148 digestion, 5% (w/w) of trypsin was added and the samples were incubated over night at 37°C. The heavy-  
149 labelled synthetic peptide was then spiked into the samples, and they were completely dried in a  
150 SpeedVac system. The samples were re-suspended in 40 µL 0.5% TFA and desalted using the SPE Pierce®  
151 C18 Spin Columns (ThermoFisher Scientific). These columns were activated by 2 x 200 µL of 50% ACN and  
152 equilibrated with 2 x 200 µL of 0.5% TFA. The tryptic peptides were adsorbed to the media using 2  
153 repeated cycles of 40 µL sample loading and the column was washed using 2 x 200 µL 0.5% TFA. Finally,  
154 the peptides were eluted in 2 x 30 µL of 70% ACN and dried. Before they were analysed on a nanoLC-  
155 LTQ-Orbitrap mass spectrometer, the peptides were re-suspended in 160 µL of 0.1% FA in Milli-Q water.

#### 156 **NanoLC-Q Exactive Plus-PRM analysis**

157 The PRM analysis was performed on a Q Exactive Plus Orbitrap mass spectrometer (ThermoFisher  
158 Scientific). An EASY-nLC 1000 system (ThermoFisher Scientific) was used for the peptide separation. A  
159 volume of 4 µL of sample was loaded onto a pre-column (EASY-Column, 2 cm, inner diameter 100 µm, 5  
160 µm, C18-A1, ThermoFisher Scientific) at a maximum pressure of 280 bar. The peptides were then eluted  
161 onto an EASY-column, 10 cm, inner diameter 75 µm, 3 µm, C18-A2 (ThermoFisher Scientific), which was  
162 used for the separation. A flow rate of 250 nL/min using mobile phase A (Milli-Q water with 0.1% FA) and  
163 B (ACN with 0.1% FA) was set for the separation. A 40 minutes gradient from 5% B to 40% B followed by  
164 10 minutes from 40% B to 75% B, and a washing step with 10% B for 10 min was used. The system was  
165 controlled through Q Exactive Plus Tune 2.5 and Xcalibur 3.0. The PRM method combined two scan  
166 events starting with a full scan event followed by targeted MS/MS for the doubly and/or triply charged  
167 precursor ion scheduled in an inclusion list. The full scan event employed a m/z 300-800 mass selection,  
168 an Orbitrap resolution of 140,000 at m/z 200, a target automatic gain control (AGC) value of  $3 \times 10^6$ , and  
169 maximum fill times of 250 ms. The targeted MS/MS was run at an Orbitrap resolution of 35,000 at m/z

170 200, target AGC value of  $1 \times 10^6$ , and maximum fill times of 200 ms. The targeted peptides were isolated  
171 using a 1.2 m/z unit window. MS/MS fragmentation was performed using the high energy collision  
172 dissociation (HCD) mode, with normalized collision energy (NCE) of 27 eV. The mass spectrometry  
173 proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>51</sup> partner  
174 repository with the data set identifier PXD009277.

#### 175 **Determination of linearity, repeatability and limits of detection (LOD) and quantification (LOQ) of the** 176 **method**

177 The IGF-II and IGFBP-3 QPrEST™ proteins and the heavy-labelled GPETLCGAELVDALQFVCGDR peptide  
178 were used in a spike-in experiment to determine the linearity and the repeatability of the targeted  
179 proteins, applying the PRM-method. For the calibration curves, the QPrEST™ were spiked into canine  
180 sera to final concentrations of 0.058, 0.29, 1.4, 7.2 and 36 fmol/μL for IGF-II, and 0.019, 0.094, 0.47 and  
181 2.3 fmol/μL for IGFBP-3. The samples were then digested and thereafter, the  
182 GPETLCGAELVDALQFVCGDR peptide was spiked into the sample to final concentrations of 0.0024, 0.012,  
183 0.059, 0.29 and 1.5 fmol/μL. These samples were analysed in triplicate. For the intraday repeatability  
184 study, three serum samples (technical replicates) from the NICTH dog after surgical removal of the  
185 splenic tumor were spiked-in with the QPrEST™ or the heavy GPETLCGAELVDALQFVCGDR peptide the  
186 same day. The concentrations were selected based on the ratios obtained in the calibration curves  
187 between the native and the synthetic peptides, to obtain an approximate ratio of 1. The final  
188 concentrations were 7.2 fmol/μL for IGF-II, and 0.47 fmol/μL for IGFBP-3. These samples were then  
189 digested and the GPETLCGAELVDALQFVCGDR peptide was spiked-in to a final concentration of 0.29  
190 fmol/μL. These samples were analysed in triplicate, and all data were processed together (n=9). For the  
191 interday repeatability study, the same sample preparation was applied as for the intraday repeatability  
192 study, but in this case, serum from the dog before and after the surgery of the splenic tumor was used.

193 The same sample was prepared in three different and non-consecutive days, and they were analysed in  
194 triplicate (n=9). For data analysis, the SkyLine 3.7.0 software<sup>52</sup> was applied to extract area under curve  
195 (AUC) of the fragments. For the statistical analysis, the built-in tool Data Analysis in Microsoft Excel  
196 Professional 2010 was used. Ordinary least squared regressions along with ANOVA were used to  
197 evaluate the fitness of the calibration curves (one predictive variable), the relation between IGF protein  
198 concentrations and weight (two variables), and the relation between IGF-I concentrations calculated  
199 using the PRM MS-based method and the ELISA method (two variables). To evaluate the effect of the  
200 splenic tumor removal in the concentration of IGF proteins, the normal distribution of the data was  
201 verified by visual examination and by the Anderson-Darling normality test, and a paired sample T-test  
202 was applied. In all these analyses, p-values < 0.05 were considered statistically significant. The LOD and  
203 LOQ for IGF-I, IGF-II and IGFBP-3 were calculated using the following formulas:  $LOD = 3S_a/b$ ,  $LOQ =$   
204  $10S_a/b$ , where  $S_a$  is the standard deviation of the minimum detectable concentration with an  $RSD \leq 20\%$ ,  
205 and  $b$  is the slope of the standard dilution curve.<sup>53</sup> LOD for big IGF-II was determined by manual  
206 inspection of analysed samples in the low concentration range, as described in the results.

## 207 **Method comparison**

208 Measurements of IGF-I is used routinely for diagnosis of GH-related diseases in dogs. Seven samples  
209 were analysed both with MS and an IGF-I ELISA (Mediagnost, Reutlingen, Germany) previously validated  
210 for use in dogs.<sup>54</sup>

211

## 212 **RESULTS**

### 213 **Internal standard selection**

214 The first step for the optimization of the targeted MS-based method used for quantification of IGF-I, IGF-  
215 II and IGFBP-3, and the incompletely processed form of IGF-II (big IGF-II), was the selection of the  
216 internal standard. IGF-I protein from dog is well defined in the UniProt database, but IGF-II and IGFBP-3  
217 are classified as “uncharacterized proteins”, with three different identifiers for IGF-II (J9NYS6, F1PBX5  
218 and 9P961) and one identifier for IGFBP-3 (F1PQ91). To study if the human-derived QPrEST™ could be  
219 used as internal standard, the canine sequences of those identifiers were aligned with the human  
220 sequences (**Figure 1**). The chosen sequences were verified as unique to the targeted proteins.



221 **Figure 1.** Homology study of IGF-I, IGF-II and IGFBP-3 dog proteins and their human analogues (\* indicates the same amino acid). The shorter  
222 mature protein is underscored. The tryptic peptides in QPrEST™ or the heavy-labelled synthetic peptide matching peptides found in the canine  
223 sequence and used for quantification are marked in bold. In grey, is marked a QPrEST™ tryptic peptide that could be theoretically used for big  
224 IGF-II quantification.

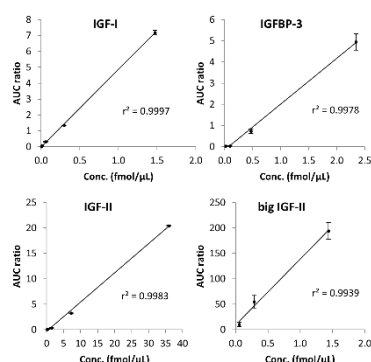
225 For IGF-I, while the sequences of the pre-processed form differ between both species (195 aa in humans  
226 vs 153 aa in dogs), the mature form of (70 aa) is identical. However, human QPrEST™ proteins that match  
227 this sequence are not available. Therefore, as was done in a previous study with cats,<sup>38</sup> a synthetic heavy  
228 isotope-labelled peptide (GPETLCGAELVDALQFVCGDR) was used for the quantification. For IGF-II, the  
229 mature form (67 aa in humans) differs in comparison with the three UniProt canine identifiers, as well as  
230 the E-peptide (or pre-processed) sequence (89 aa in humans). Nevertheless, some regions are conserved,  
231 and the same QPrEST™ used in our previous study was selected. This QPrEST™ protein contains five  
232 tryptic peptides that match well with the three canine IGF-II identifiers. Two of these peptides,

234 GIVEECCFR and SCDLALLETYCATPAK, match perfectly with a region of the mature IGF-II. However, when  
235 a shotgun analysis of the QPrEST™ standard was performed, a miss-cleavage in SCDLALLETYCATPAK  
236 peptide but not in GIVEECCFR peptide was observed; therefore the latter was selected to increase the  
237 reliability of the quantification. The other three peptides of the QPrEST™ protein match with  
238 unprocessed forms of IGF-II. Among them, GLPALLR peptide matches with the pro-IGF-II obtained after  
239 the cleavage of a 24 amino acid signal peptide (known as IGF-2[1-156]). However, this peptide was not  
240 found in the shotgun analysis of the QPrEST™ standard. Another peptide is DVSTPPTVLPDNFPR, which  
241 matches with one of the big IGF-II forms (known as big IGF-2[1-87]). In this case, a miss-cleavage of the  
242 peptide was observed in the shotgun analysis, and it was discarded for the quantification. The last  
243 peptide is FFQYDTWK, which matches with the big IGF-II form known as [1-104]. This peptide was found  
244 in the shotgun analysis without any miss-cleavage, therefore it was chosen to quantify the levels of big  
245 IGF-II. For IGFBP-3, the human and the reported canine sequences have the same length (291 aa) but  
246 they differ in 20% of the aa. In spite of these differences, the QPrEST™ contains a tryptic peptide that  
247 matches well with the F1PQ91 identifier, and it was selected for IGFBP-3 quantification.

## 248 **Method evaluation**

249 The parameters of the Q Exactive Plus Orbitrap MS were similar to those used in our previous study,<sup>43</sup>  
250 but we also included the analysis of a peptide that matches with the big IGF-II form [1-104]. The final  
251 settings for the method can be found in the material and methods section. The retention times of the  
252 selected peptides were confirmed using a shotgun analysis, and an inclusion list with the native and the  
253 heavy isotope-labelled peptides was included in the PRM-method (see **Table 1**). For IGF-I and IGF-II  
254 peptides, six MS/MS-fragments were used for quantification, and for big IGF-II and IGFBP-3, three  
255 MS/MS-fragments were used. After the selection of the PRM-method parameters, evaluation of the  
256 linearity, the repeatability and the LOD and LOQ was performed. Linear regression of the normalized

257 signals to the internal standard peptides over the entire investigated ranges resulted in determination  
 258 coefficients of 0.9997 ( $p < 0.001$ ), 0.9978 ( $p < 0.001$ ) and 0.9983 ( $p < 0.001$ ) for IGF-I, IGFBP-3 and IGF-II  
 259 (**Figure 2**, Raw data and calculations are given in **Supporting Table S1**). In the case of big IGF-II, linearity  
 260 was observed in the range 0.058 - 1.4 fmol/ $\mu$ L, resulting in a determination coefficient of 0.9939  
 261 ( $p < 0.001$ ).



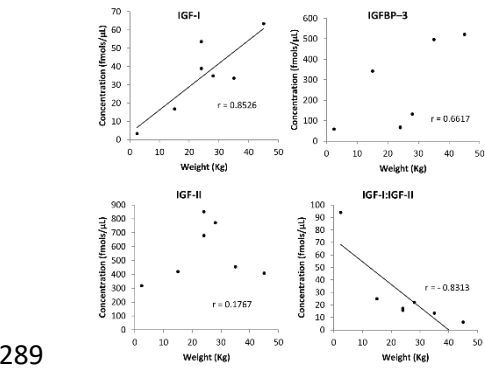
262 **Figure 2.** Calibration curves for IGF-I, IGFBP-3, IGF-II and big IGF-II dog proteins. The ratio synthetic/native peptide is plotted against the spiked  
 263 concentration.  
 264

265 For the repeatability study, the retention times of the selected peptides, and the quantification based on  
 266 the normalized AUC signals were used (**Tables 2 and 3**, Raw data and calculations are given in **Supporting**  
 267 **Table S2**). The data indicate that the retention times of IGFBP-3, IGF-II, big IGF-II and IGF-I, were 14, 23,  
 268 29 and 40 minutes respectively, and the combination of all data shows a RSD lower than 5%. These  
 269 results demonstrate the good repeatability of the method. In terms of quantification, the RSD values  
 270 were good for IGF-I and acceptable for the other proteins. LOD and LOQ were calculated based on a  
 271 standard statistical approach (**Supplementary Table S1**). Due to few calibration points in the linear range  
 272 in combination with very low concentrations of big IGF-II in samples from healthy dogs and in the dog  
 273 with NICTH after surgery, the LOD of big-IGF-II was estimated based on the observation of well defined  
 274 MS/MS-fragments and the standard deviation in the repeatability study (**Table 3**). In this sample there  
 275 were at least two well defined MS/MS-fragments in all intraday measurements and at least one well

276 defined fragment in all interday measurements. In addition, we considered RSD on the interday  
277 measurements to be acceptable. The LOD was estimated to be 6.59 fmol/μL. The LOQ was not estimated  
278 for this protein.

279 **Quantification of IGF-I, IGF-II, big IGF-II and IGFBP-3 in dogs**

280 The concentrations of IGF-I, IGF-II, big IGF-II and IGFBP-3 were determined in serum from 7 healthy dogs  
281 (**Table 4**, Raw data and calculations are given in **Supporting Table S3**). Due to the low concentrations of  
282 big IGF-II in healthy dogs two healthy dogs were below LOD. All samples were analysed in two replicates  
283 using the developed PRM-method. The measurements gave RSD values similar as those observed in the  
284 repeatability study., As expected based on previous studies,<sup>43,44</sup> there was a significant correlation  
285 between weight and IGF-I ( $r = 0.8526$ ,  $p < 0.05$ ) (**Figure 3**). There was no correlation between weight and  
286 IGF-II ( $r = 0.1767$ ,  $p = 0.705$ ) and the trend to a correlation between weight and IGFBP-3 did not reach  
287 statistical significance ( $r = 0.6617$ ,  $p = 0.105$ ). The IGF-II:IGF-I ratio was inversely correlated to weight ( $r = -$   
288  $0.8313$ ,  $p < 0.05$ ).



290 **Figure 3.** Regression curves obtained after plotting the quantified concentrations of IGF-I, IGF-II and IGFBP-3 proteins, and the IGF-II: IGF-I ratio  
291 against the weight of seven dog samples serum.

292 **Method comparison**

293 There was good agreement between IGF-I concentrations calculated using the PRM MS-based method  
294 and the ELISA method ( $r = 0.9254$ ,  $p < 0.05$ ) (Raw data and calculations are given in **Supporting Table S4**).



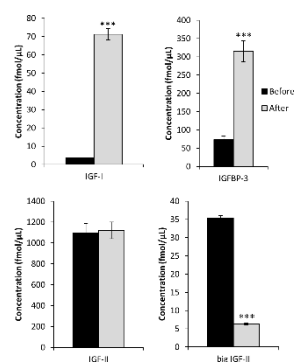
295

296 **Canine case study of NICTH syndrome**

297 A 9-year old male Labrador retriever, weighing 40 kg, was admitted to a large Veterinary Hospital in  
298 Stockholm, Sweden, with sudden ataxia and weakness. At presentation, the dog was unable to stand up  
299 and was treated as an in-house patient. Body temperature, heart and pulmonary auscultation were  
300 normal. No abnormal findings were detected on neurologic exam but postural reactions were difficult to  
301 assess due to difficulties of standing.

302 Serum and EDTA-blood were submitted to the laboratory for a basic panel. The results revealed  
303 profound hypoglycemia, low insulin concentrations and very mild increase in sodium (**Supporting Table**  
304 **S5**). The dog was treated with i.v. fluids containing glucose and despite this there was persistent  
305 hypoglycemia (2.1, 1.2, 1.4 and 3.4 mmol/L, reference interval 3.7-6.6) although the signs of weakness  
306 and ataxia disappeared with glucose infusion. The dog received one injection of prednisolone (1 mg/kg  
307 sc) which was followed by oral administration (1 mg/kg once daily) the next day. Ultrasound revealed an  
308 11 x 15 cm mass in the spleen. There were no abnormal findings in the pancreas. No signs of metastasis  
309 were found on radiographs of lungs. The spleen was removed and sent for histopathology and the dog  
310 was sent home on prednisolone and frequent meals. Histopathology demonstrated a leiomyoma. At re-  
311 check one week after the tumor was removed, the dog was euglycemic (5.9 mmol/L) and had no  
312 abnormal clinical signs. Prednisolone was tapered and withdrawn. At recheck 3 months post-surgery, the  
313 dog was still euglycemic and insulin had increased to 200 ng/L which was within reference interval (40-  
314 380 ng/L). Based on hypoglycemia and low insulin concentration together with a splenic tumor, NICTH  
315 was suspected. In humans with NICTH, feedback inhibition by increased concentrations of big IGF-II will  
316 usually induce a pattern with low IGF-I and IGFBP-3. The concentrations of IGF proteins were determined  
317 in this dog. As shown in **Figure 4**, there was a significant increase of IGF-I (from 3.73 to 71.2 fmol/ $\mu$ L,

318 p<0.001) and IGFBP-3 (from 73.6 to 315 fmol/μL, p<0.001) between samples taken before and 3 months  
 319 after tumor removal off any medications. Concentrations of big IGF-II decreased by 80% (from 35.4 to  
 320 6.59 fmol/μL, p<0.001) whereas concentrations of total IGF-II did not change significantly (p=0.85).



321  
 322 **Figure 4.** Mean concentration and standard error of the mean of IGF-I, IGFBP-3, IGF-II, big IGF-II proteins in a dog with NICTH syndrome, before  
 323 and after the removal of the splenic tumor (\*\*\*) indicates significant differences after a two-sample T-test, p-value < 0.001).

## 325 DISCUSSION

326 Measurement of IGF proteins in dogs using RIA, ELISA or WLB possess limitations which may be  
 327 overcome using MS-based methodologies.<sup>55</sup> In our study, a previously developed PRM-method for cats  
 328 has been optimized and applied to quantify four canine members of the IGF system (IGF-I, IGF-II, big IGF-  
 329 II [1-104] and IGFBP-3) in healthy dogs, and in a dog with NICTH syndrome, a rare paraneoplastic  
 330 phenomenon. To the best of our knowledge, this is the first time that simultaneous MS measurements of  
 331 the IGF system proteins have been performed in serum from a dog with NICTH, and the first time that  
 332 big IGF-II [1-104] has been measured by mass spectrometry in any species. The targeted amino acid  
 333 sequences were identical in humans and dogs, and it is therefore possible that the method can also be  
 334 applied in humans with NICTH syndrome.

335 The developed method allows the measurement of all proteins at the same time, decreasing the  
336 variation due to the methodology (IGFBPs are not interfering in the assay), and reducing the amount of  
337 serum needed for the analysis (less than 1  $\mu$ L). In addition, the high sensitivity obtained by using a  
338 nanoLC coupled to a high-resolution Q Exactive Plus Orbitrap MS method reduced the tedious and time-  
339 consuming sample preparation steps needed to increase the sensitivity of other methods,<sup>28,31,32</sup> and  
340 more proteins can easily be added to the list of proteins to be quantified if suitable peptides for  
341 quantification are identified. Moreover, QPrEST™ synthetic proteins were used for the quantification of  
342 IGF-II, IGFBP-3 and big IGF-II, and they were added to the samples prior to the digestion step. As far as  
343 we know, we are the first group using QPrEST™ human proteins to quantify non-human proteins,  
344 previously in cats,<sup>43</sup> and now in dogs. QPrEST™ synthetic proteins are well characterized, they are cheap  
345 to produce and purchase, and they offer extensive human proteome coverage. However, the use of  
346 QPrEST™ has the limitation that, in general, fewer tryptic peptides can be selected for quantification. In  
347 the present study, several tryptic fragments were available for IGFBP-3, IGF-II, and big IGF-II  
348 quantification, but only one peptide for each protein met all the requirements needed for a reliable  
349 quantification. In addition, no QPrEST™ proteins were available to target the active sequence of IGF-I,  
350 therefore an isotope-labelled tryptic peptide was used. The peptide chosen for IGF-I quantification  
351 (GPETLCGAELVDALQFVCGDR) has been used in previous works to quantify IGF-I protein in human,<sup>28,33,56</sup>  
352 and cat samples<sup>43</sup> with successful results. This peptide contains a proline in its sequence, which might  
353 induce the “proline effect”, characterized by favouring the cleavage of the N-terminal to proline during  
354 MS/MS fragmentation. However, this effect is mainly observed when the collision-induced dissociation  
355 (CID) fragmentation mode is used and we used the high energy collision dissociation (HCD)  
356 fragmentation mode. HCD has no low-mass cutoff, higher resolution and, because it employs higher  
357 energy dissociations than CID, it enables a wider range of fragmentation pathways. In addition, the  
358 selected peptide contains glutamine, which is prone to deamidation. This modification was observed in

359 the preliminary shotgun analysis, but its abundance was very low compared to the unmodified form. In  
360 the case of IGFBP-3, the selected peptide has been used to quantify the levels of IGFBP-3 protein in  
361 cats,<sup>43</sup> and our data also demonstrate good linearity and acceptable repeatability based on the RSD  
362 value. The high RSD value could be due to the small amount of this protein in the serum, but also  
363 because of the chemical properties of the peptide used. This peptide is hydrophilic and it can be lost  
364 differentially during the desalting process, or because of the instability of the electrospray at early time  
365 points (it elutes early in the gradient, minute 14, and it has the highest RSD of all peptides used). Some  
366 difficulties have previously been seen when analysing IGFBP-3 protein by mass spectrometry.<sup>34</sup> The  
367 authors of that work suggested that the problems could be due to the formation of complexes between  
368 large molecular weight proteins from plasma and IGFBP-3, and they developed a labour intensive sample  
369 preparation protocol including precipitation and delipidation steps to concentrate the targeted proteins.  
370 However, the authors still had accuracy problems for the quantification of IGFBP-3. In the present work,  
371 we aimed to develop a simple and rapid method that require less than 1  $\mu$ L of serum. The peptide  
372 selected for the quantification of IGF-II has been previously used to quantify the levels of IGF-II protein in  
373 humans,<sup>34</sup> and cats,<sup>43</sup> and our data also demonstrates good linearity and acceptable repeatability. Finally,  
374 the peptide FFQYDTWK was selected to quantify the levels of big IGF-II form, and this peptide has never  
375 been used either in humans or dogs.

376 In humans, increased concentration of bioavailable IGF-II can cause negative feedback on pituitary GH  
377 secretion, leading to low IGF-I and IGFBP-3 concentrations.<sup>1</sup> Based on that, the recommendation is to  
378 consider the diagnosis of NICTH in a patient if hypoglycemia, suppressed insulin, and low IGF-I  
379 concentrations are found.<sup>57</sup> In dogs, at profound low glucose concentrations, insulin should be low or  
380 undetectable<sup>20</sup> and this is an important difference when separating NICTH from insulinoma, where  
381 hypoglycemia is accompanied by normal to high insulin concentrations. However, there are to date no  
382 previous case reports of canine NICTH where both insulin and IGF-I have been measured. Therefore, the

383 application of the present method to measure the levels of IGFs proteins in a dog with NICTH syndrome  
384 has revealed that IGF-I was decreased, which together with the low insulin levels, suggest the  
385 recommendations made for human NICTH are also applicable in dogs.

386 Moreover, the ratio of IGF-II:IGF-I >10 has been suggested as an additional marker for diagnosis of NICTH  
387 in humans.<sup>57</sup> In our study, the IGF-II:IGF-I ratio was inversely correlated to weight. This may be explained  
388 by differences of the IGF-system. In dogs, we and others<sup>22,48,49</sup> have observed that IGF-I concentrations,  
389 but not IGF-II, are strongly associated with body size. This finding will impact the IGF-II:IGF-I ratio. In our  
390 study, the smallest dog (2.4 kg) had an IGF-II:IGF-I ratio of 94, whereas the largest dog (46 kg) had a ratio  
391 of 6. The dog with NICTH syndrome weighed approximately 40 kg and the IGF-II:IGF-I ratio decreased  
392 from 294 to 16 after removal of the tumor. Dogs were of different breeds, ages and gender but  
393 unfortunately there were too few dogs to investigate possible confounding factors. Measurements from  
394 a larger population of healthy dogs of different sizes are needed before any ratio between IGF-II and IGF-  
395 I can be recommended as an additional marker for canine NICTH.

396 Furthermore, in human patients with NICTH, total IGF-II concentrations measured by immunoassays are  
397 reported to be low, within RI or high.<sup>1</sup> NICTH in humans is often caused by precursor forms of IGF-II,  
398 usually big IGF-II [1-87] or IGF-II [1-104], and for diagnosis with immunoassays, antibodies targeting  
399 these big forms are needed. Big IGF-II represents at least 10% of total IGF-II in the healthy population,<sup>58</sup>  
400 and this proportion is increased with NICTH.<sup>59</sup> As well as for IGF-II, there are no standard methods to  
401 quantify big IGF-II [1-104] in dogs. Here we present a novel MS-based approach. In serum from the dog  
402 with NICTH, the concentration of the protein before surgery was determined with high confidence and  
403 an RSD value of  $\approx 5\%$  (**Table 3**). Values decreased considerably after tumor removal and was similar to  
404 healthy dogs. LOD was calculated based on the sample from the dog with NICTH after surgery and was  
405 6.59 fmol/ $\mu$ l. This assumption restricts the calculations of the absolute concentrations in some healthy

406 dogs. The low concentrations in healthy dogs and the relatively high RSD seen at low concentrations of  
407 big IGF-II and IGFBP-3 indicates that one should be cautious about interpreting small changes of these  
408 proteins with this method. Based on biological variation of IGF-I in dog, RSD up to 10.5% is considered  
409 desirable and up to 15.7% is considered acceptable.<sup>60,61</sup> All the measurements of IGF-I in the present  
410 study demonstrated an RSD of <12.7%. To the best of our knowledge, there are no expert opinions or  
411 data on biological variation to help determine a clinically acceptable RSD for big IGF-II, IGFBP-3 and total  
412 IGF-II in dogs. Further studies where these proteins are measured in healthy dogs as well as in diseased  
413 dogs are needed to evaluate the clinical usefulness of this method. Before surgery, big IGF-II [1-104] was  
414 only 3.23% of total IGF-II, which is lower than previous reports in human serum. It is possible that other  
415 precursor forms of IGF-II (e.g. big IGF-II [1-87]) are present, and this could explain why big IGF-II [1-104]  
416 was such a small percentage of the total IGF-II. Another interesting aspect is that the sequence used for  
417 quantifying big IGF-II [1-104] is located in the E-peptide region and the cleaved E-peptide can circulate in  
418 serum as well as being a part of big IGF-II.<sup>59</sup> In the study by Daughaday and Trivedi, immunoreactivity  
419 against the cleaved IGF-II E-peptide was similar in serum from healthy subjects compared to a NICTH  
420 patient.<sup>59</sup> The cleaved E-peptide has been named preptin and found to be co-secreted with insulin by  
421 pancreatic beta-cells.<sup>62</sup> There is scarce information about preptin but, as reviewed by Aydin in humans  
422 and rats, it seems to be mainly secreted from pancreas and is positively associated with insulin  
423 concentrations.<sup>63</sup> In the dog with NICTH syndrome, undetectable insulin concentrations, and high big  
424 IGF-II [1-104] concentrations were found before surgery, while insulin increased and big IGF-II [1-104]  
425 decreased after surgery. If dogs show the same patterns as humans and rats, most of the measured  
426 sequence is likely to represent big IGF-II. However, further studies are needed to evaluate preptin and its  
427 role in the IGF-system.

428

429 **CONCLUSION**

430 NICTH is a rare paraneoplastic syndrome and should be suspected in dogs with persistent hypoglycemia,  
431 in the presence of low insulin and low IGF-I. High precursor forms of IGF-II would further support  
432 diagnosis. We have developed a PRM MS-based method that allows the simultaneous quantification of  
433 IGF-I, total IGF-II, IGFBP-3 and, for the first time, the quantification of big IGF-II protein in dogs. This  
434 method overcomes the need for antibody availability or potential antigen cross-reactions that are  
435 problems in enzymatic or radioimmunoassays, as well as the tedious and time consuming sample  
436 preparation. It is also adaptable and scalable to detect other biomarkers of interest if suitable peptides  
437 for quantification are identified, and it requires less than 1  $\mu$ L of serum, making this method potentially  
438 useful for clinical laboratories. Moreover, we have used the heavy-labelled synthetic QPrEST™ proteins  
439 designed for humans, which enable the correction for possible incomplete proteolysis that could affect  
440 the selected peptide and cause variability in the protein quantification. The method was demonstrated  
441 to possess good linearity and repeatability, and has been successfully applied to a dog with NICTH  
442 syndrome, with results in accordance with previous human studies found in the literature. These  
443 measurements may aid in diagnosing NICTH, however further studies with more dogs are needed to  
444 evaluate reference ranges and diagnostic performance of this method.

445

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449

450    **Conflict of interest**

451    The authors declare no competing financial interest.

452



453 **Figure legends**

454 **Figure 1.** Homology study of IGF-I, IGF-II and IGFBP-3 dog proteins and their human analogues (\*  
455 indicates the same amino acid). The shorter mature protein is underscored. The tryptic peptides in  
456 QPrEST™ or the heavy-labelled synthetic peptide matching peptides found in the canine sequence and  
457 used for quantification are marked in bold. In grey, is marked a QPrEST™ tryptic peptide that could be  
458 theoretically used for big IGF-II quantification.

459 **Figure 2.** Calibration curves for IGF-I, IGFBP-3, IGF-II and big IGF-II dog proteins. The ratio  
460 synthetic/native peptide is plotted against the spiked concentration.

461 **Figure 3.** Regression curves obtained after plotting the quantified concentrations of IGF-I, IGF-II and  
462 IGFBP-3 proteins, and the IGF-II:IGF-I ratio against the weight of seven dog serum samples.

463 **Figure 4.** Mean concentration and standard error of the mean of IGF-I, IGFBP-3, IGF-II, big IGF-II proteins  
464 in a dog with NICTH syndrome, before and after the removal of the splenic tumor (\*\*\*) indicates  
465 significant differences after a two-sample T-test, p-value < 0.001).

466 **Tables**

467 **Table 1.** Targeted peptides included in the PRM method.

Protein	[m/z] (Th)	Charge	Sequence	Position	RT (min)	Fragments
IGF-I	769.6963	3+	GPETL <u>C</u> GAELVDALQFV <u>C</u> GDR (light)	1-21	40.3	Y <sub>4</sub> , Y <sub>5</sub> , Y <sub>6</sub> , Y <sub>7</sub> , Y <sub>10</sub> , b <sub>9</sub>
	773.0324	3+	GPETL <u>C</u> GAELVDALQFV <u>C</u> GDR (heavy)	1-21	40.3	
IGF-II	585.2575	2+	GIVEECCFR (light)	41-49	22.5	Y <sub>3</sub> , Y <sub>4</sub> , Y <sub>5</sub> , Y <sub>6</sub> , Y <sub>7</sub> , b <sub>3</sub>
	590.2617	2+	GIVEECCFR (heavy)	41-49	22.5	
big IGF-II	567.7664	2+	FFQYDTWK (light)	90-98	29.4	Y <sub>5</sub> , Y <sub>6</sub> , Y <sub>7</sub> ,
	571.7735	2+	FFQYDTWK (heavy)	90-98	29.4	
IGFBP-3	506.2136	2+	ETGYGPCR (light)	207-214	14.2	Y <sub>2</sub> , Y <sub>4</sub> , Y <sub>5</sub>
	511.2178	2+	ETGYGPCR (heavy)	207-214	14.2	

468 C-terminal arginine (R) and Lysine (K) of heavy peptides were labelled with (<sup>15</sup>N , <sup>13</sup>C). All cysteines are carbamidomethylated  
469 (underscored).

470

471 **Table 2.** Intraday repeatability data of the peptides in dog’s serum after surgery. Three serum samples  
472 (technical replicates) were prepared independently and each sample was analysed in triplicate (n=9).

	Retention time		Quantification		
	Mean (min)	RSD (%)	Mean (fmol/μL)	SEM (fmol/μL)	RSD (%)
IGF-I	41.0	0.33	79.1	1.08	4.11
IGF-II	23.1	1.7	977	68.3	19.8
big IGF-II	30.1	1.1	5.49	0.555	26.7
IGFBP-3	14.6	2.8	312	34.9	33.6

473

474

24

475 **Table 3.** Interday repeatability data of IGF-peptides in a case of canine NICTH before and after surgery.  
476 The same sample was prepared in three different and non-consecutive days, and they were analysed in  
477 triplicate (n=9).

	Before surgery, n=9					After surgery, n=9				
	Retention time		Quantification			Retention time		Quantification		
	Mean	RSD	Mean	SEM	RSD	Mean	RSD	Mean	SEM	RSD
	(min)	(%)	(fmol/μL)	(fmol/μL)	(%)	(min)	(%)	(fmol/μL)	(fmol/μL)	(%)
IGF-I	40.2	1.6	3.73	0.106	8.08	39.8	2.4	71.2	3.01	12.7
IGF-II	22.4	3.5	1100	88.7	24.3	22.0	4.2	1120	79.8	21.4
big IGF-II	29.3	3.0	35.4	0.651	5.20	28.7	4.2	6.59	0.313	14.3
IGFBP-3	14.2	4.2	73.6	8.69	33.4	13.7	4.9	316	28.6	25.7

478

479 **Table 4.** Weight and quantified levels of IGF proteins in 7 different dogs.

	Weight	IGF-I		IGF-II		IGFBP-3		big IGF-II		IGF-II:IGF-I ratio	
		Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
	(Kg)	(fmol/μL)	(%)	(fmol/μL)	(%)	(fmol/μL)	(%)	(fmol/μL)	(%)		(%)
Dog 1	24	39.0	3,70	681	4,92	66,2	28.0	9,68	29,3	17,5	8,61
Dog 2	24	53.7	0,603	852	8,94	69,5	12,4	13,3	3,95	15,9	8,34
Dog 3	2.4	3.39	6,19	319	12.0	59,3	51,2	<6.59 <sup>a</sup>	-	94,6	18,2
Dog 4	28	35.0	0,456	773	11,7	133	27.0	11,4	27,2	22,1	11,3
Dog 5	45	63,6	4,85	410	4,11	522	9,75	15	3,99	6,44	0,738
Dog 6	35	33,7	1,80	455	1,21	498	10,9	9,48	1,24	13,5	0,587
Dog 7	15	16,8	0,343	420	3,81	343	1,22	<6.59 <sup>a</sup>	-	25.0	3,47

480 <sup>a</sup> Concentration below the estimated LOD.

481 **Supporting Information**

482

483 **Supporting Table S1.** Raw data and calculations for the construction of the linear regression.

484 **Supporting Table S2.** Raw data and calculations for intraday and interday repeatability study.

485 **Supporting Table S3.** Raw data and calculations for the quantification of IGF proteins in 7 different dogs.

486 **Supporting Table S3.** Raw data and calculations for the correlation between IGF-I concentration  
487 calculated using the PRM MS-based method and the ELISA method.

488 **Supporting Table S5.** Biochemistry and hematological results at presentation.

489

490 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via  
491 the PRIDE partner repository with the dataset identifier PXD009277.

492 Reviewer account details:

493 Username: reviewer65234@ebi.ac.uk

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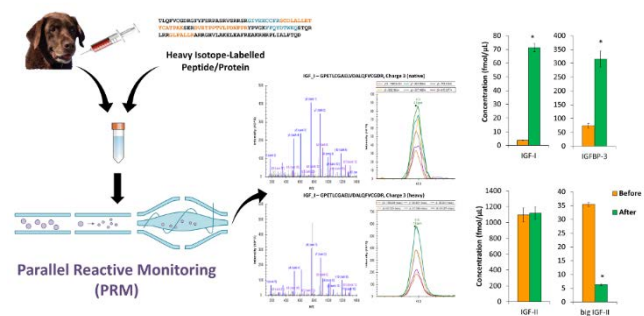
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